During the PD-125402 grant period the automaticity of the sinus-node was investigated. Our research primarily focused on the NCX function in the spontaneous pacemaking. In the last 20 years the crucial role of the NCX in sinus node pacemaking was firmly proved experimentally as well as by numerical computational models. Based on our current understanding, the sinus-node pacemaking is a highly complex machinery where both transmembrane ionic currents (such as If, or ICaL, ICaT, IKr) and the local Ca2+ release-mediated NCX inward current have important role. These processes contribute to the spontaneous pacemaking, providing a robust, flexible, and fail-safe mechanism (coupled-clock).

Even though the high number of experimental evidences, there is a lack in the literature regarding the effect of selective NCX inhibition in the sinus-node, therefore a direct pharmacological evidence is still missing.

The main aims of the project were the followings:

- Has any effect of the selective NCX inhibition on the pacemaker frequency of the rabbit sinus node?
- Estimation the cooperation between the ionic currents of the pacemaker mechanism: the role of I_{Kr}, I_{NCX}, $[Ca^{2+}]_i$ and I_f
- Has any effect of the selective NXC inhibition on the Ca^{2+} transients in isolated sinus node cells?

I.) In order to address these issues, it was crucially important to use the available most selective NCX inhibitor. In our previous study the ORM-10962 was found to be completely selective to the NCX (Kohajda et al, 2016, Plos One). In order to better understand the effect of ORM-10962, prior to the sinus-node experiments we performed a detailed study to investigate the degree of NCX inhibition during intact Ca handling by using 1 uM ORM-10962. Our results showed that the effect of ORM on NCX is reduced when the Ca homeostasis is intact compared with conventional protocols during buffered Ca (55 % vs 90%). This effect could be the consequence of the autoregulation of the Ca handling and/or the asymmetrical inhibition of the reverse and forward mode (i.e. reverse mode inhibition is higher) and/or the preserved inducibility of the NCX by the increased intracellular Ca.

This study provided important results indicating that under normal condition, and intact Ca-handling the available NCX inhibition is nearly 50% when 1 uM ORM-10962 is applied (*Oravecz et al, 2018, EJP*).

II.) In the second part of the project we applied the selective NCX inhibitor ORM-10962 on sinus-node tissue and isolated cells. Our experimental and in-silico modelling data revealed the following:

(i) Inhibition of the NCX by 1 uM ORM-10962 caused only a marginal but statistically significant cycle length increase (cca. 8%)

(ii) this effect was coupled with a large increase of the Ca2+I, that could counteract to the cycle length prolonging effect of selective NCX inhibition via inducing Ca-dependent inactivation of ICaL, and activating the Ca-dependent potassium channels. Therefore, it is feasible that the observed moderate effect of NCX on cycle length is the consequence of several direct and indirect mechanisms.

(iii) Combined inhibition of funny current (If) and NCX revealed a close cooperation between currents providing a reserve capacity for the diastolic depolarization.

(iv) Inhibition of the I_{Kr} caused a significant increase of the cycle length via prolongation of the action potential duration. The I_{Kr} provided to be independent from the If-NCX cooperation

(v) The cooperative function of the If-NCX has an important role in the cycle length stability as well. Similarly to the cycle length, the beat-to-beat cycle length variability was also largely increased when these current were inhibited.

Our results strongly suggest a "pacemaker reserve" working during the diastolic depolarization which means a close cooperation between NCX and I_f. This “crosstalk” implies that inhibition of one current (NCX of If) did not cause excessive sinus node action potential cycle length prolongation. However, when both currents are inhibited the cycle length largely increases leading to enhanced beat-to-beat cycle length variability. This indicate that cooperation between NCX and If stabilizes not only the actual cycle length but also controls the rhythm of pacemaking. Therefore, these currents may provide a safe and redundant pacemaking during various condition (*Kohajda et al, 2020, Frontiers in Pharmacology*). Previous study from our laboratory reported that selective NCX inhibition could be a promising antiarrhythmic tool against Na-induced Ca-overload mediated arrhythmias in ventricle (Nagy et al, BJP, 2014). Since these arrhythmias are often coupled with elevated heart rate, a small but statistically significant bradycardia caused by selective NCX inhibition could contribute to the arrhythmic effect in some extent.

III.) In patients with end-stage renal failure it was observed that the heart rate drops very low value before they suffer sudden cardiac death with an unexplained high incidence. It was hypothesised that the electrolyte change may cause a serious malfunction in the sinus-node pacemaking. The applied human sinus-node model revealed that change of the extracellular Ca2+ markedly affect the heart rate primarily via the attenuation of the Ca2+ current. The decrease in the Ca2+ caused a secondary change in the NCX function that ultimately led to decreased slope of diastolic depolarization and bradycardia. In conclusion, we found a mechanistic explanation for the observed hypocalcaemia induced bradycardia in renal failure patients *(Loewe et al, 2019, Biophysical Journal*).

IV.) The role of the NCX in the sinus-node pacemaking, as well as the sinus-node electrophysiology were described and discussed in two review articles (*Kohajda et al, 2020, Frontiers in Pharmacology, Varró et al, 2020, Physiological Reviews*). In these studies we described the discovery and emerging role of the NCX in sinus node pacemaking from the earliest studies to the recent findings. The underlying ionic currents of sinus node action potential, and arrhythmogenic aspects of the sinus node function was also presented in detail.

- *Has any effect of the selective NCX inhibition on the pacemaker frequency of the rabbit Purkinje fibers?*

V.) The Purkinje-fibre electrophysiology represents a distinguished area of cardiac electrophysiology since the vast majority of pioneer electrophysiological studies were performed on Purkinje fibres. In contrast, after the discovery of the funny-current, the pacemaking research was almost exclusively performed on sinus-node cells, and recently, large number of data are available for the sinus-node but the Purkinje pacemaking mechanism is much less understood. The Purkinje research was seriously hampered by the difficulties of cell isolations especially in human. Recently, our laboratory successfully measured Purkinje fibre action potentials from the human ventricle (Nagy et al, CJPP, 2014). Together with our cooperation partner in Oxford, we could analyse the Purkinje fibre automaticity by using a novel human Purkinje model. In this study we found that a delicate balance between I_f and I_{K1} determines the diastolic depolarization and any interventions or alterations influencing these

currents (electrical remodelling, hyperkalaemia) has an impact on the Purkinje-fibre pacemaking and leads to serious arrhythmogenic consequences. (*Trovato et al, 2020, Journal of Cellular and Molecular Cardiology*).

The effect of NCX inhibition was also investigated in rabbit Purkinje fibers. We found that the selective NCX inhibition by 1 uM ORM-10962 caused statistically significant cycle length prolongation (540±31 ms vs 647±55 ms, $p<0.05$, n=8). 0.5 uM ivabradine also decreased the cycle length (496±42 ms *vs* 664±51 ms, $p<0.05$, n=8). When 1 uM ORM-10962 was subsequently applied, the cycle length increased to 892±106 ms. This means that 1 uM ORM-10962 alone increased the cycle length by 23±4%, in contrast when was applied after 0.5 uM ivabradine the cycle length prolongation of 1 uM ORM-10962 was 40±7% (Figure 1).

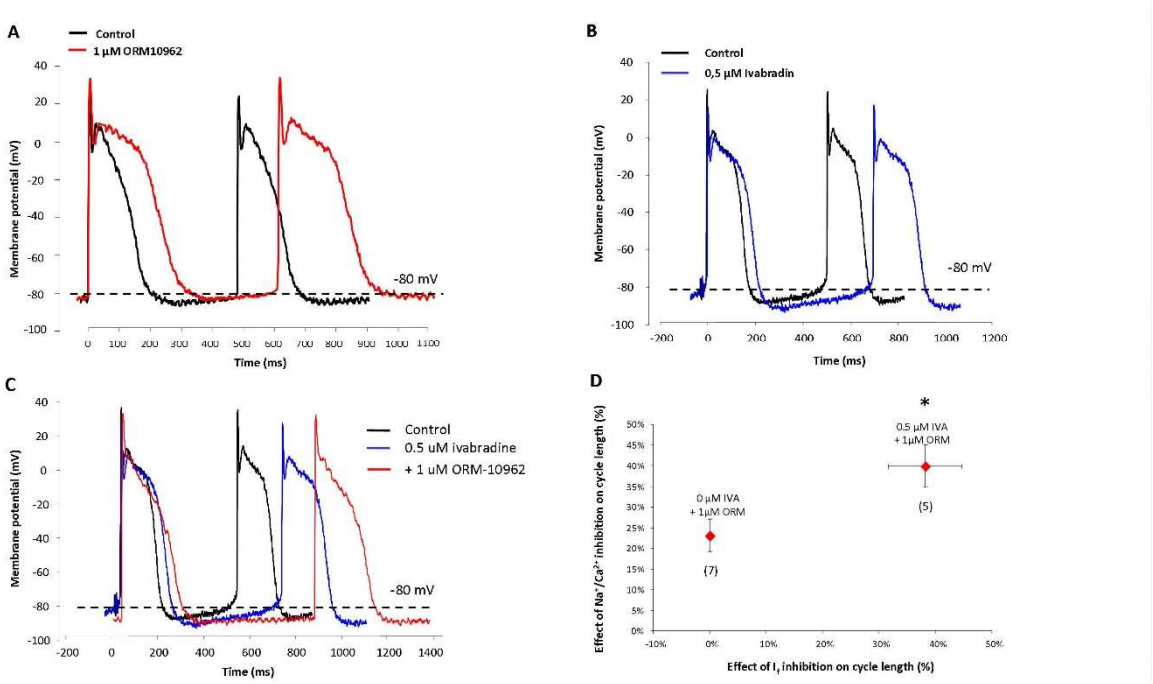

Figure 1: *Effect of selective NCX and funny-current inhibition on spontaneously beating Purkinje fibers.* ***Panel A*** *shows the effect of 1 uM ORM-10962 (red trace) while* ***panel B*** *represents the cycle length prolongation after application of 0.5 uM ivabradine (blue trace). In* ***panel C****, red trace illustrates the cumulative effect of 0.5 uM ivabradine and 1 uM ORM-10962. In* ***panel D****, effect of ORM-10962 is plotted against the effect of ivabradine. In the presence of If inhibition the effect of 1 uM ORM-10962 is nearly doubled (unpublished results).*

This indicate that NCX and If operates together in Purkinje fibre providing a redundant pacemaking mechanism. Combined with our in-silico results (*Trovato et al*) the IK1-If-NCX currents may have crucial role in Purkinje-fibre pacemaking.

- *Estimation the role of NCX in hypertrophic cardiomyopathy*

VI.) Several diseases, pathological conditions, or physiological adaptations influence the sinus-node automaticity. Among these, the excessive physical training of top athletes is known to associate with hypertrophy and sinus-bradycardia. It has critical importance, since the sudden cardiac deaths in top athletes is 2-3 times larger compared to the normal age-matched population. However, the underlying mechanism of the sinus-bradycardia is controversial in the literature and not fully clarified. The classical viewpoint claims the prominent role of the increased vagal tone, however, recent results demonstrated the importance of I_f-downregulation (D'Souza et al, Nature, 13;5:3775).

In our swimming-trained rat athlete model we observed left ventricular hypertrophy, increased ejection fraction, larger Ca content of the sarcoplasmic reticulum, and sinus-bradycardia in-vivo. However, when the hearts were monitored by ex-vivo Langendorff method (i.e. denervated condition), the sinus frequency was identical between trained and sedentary groups. These results indicate that in our model the sinus-bradycardia was the consequence of the increased vagal tone, and in our model electrical remodelling of the sinus node is not expected (*Gazdag et al, 2020, Scientific Reports*).

We aim to further investigate this issue on exercised trained dogs to obtain data that more relevant to human, especially for the repolarization process. Recently, we established the methodology of running-trained rabbit and dog athlete's heart model in our Department (*Polyák et al, 2019, Reviews in Cardiovascular Medicine*).

VII.) However, ORM-10962 is a completely selective, effective, and suitable inhibitor for NCX research, we aimed to develop novel, more potent NCX inhibitors during the grant period. Recently, we successfully developed and tested a novel compound ORM-11372 that exerts lower EC50 values than ORM-10962. Therefore, ORM-11372 could be a promising novel tool for further NCX research and as a positive inotropic agent. (*Otsomaa et al, 2020, British Journal of Pharmacology*).

Methods: In this project we applied 9 different methods to gather comprehensive data: patch-clamp technique, conventional microelectrode technique, fluorescent optical method, western-blot, qPCR, ex-vivo Langendorff perfusion, in-silico computational modelling, in-vivo ECG and ultrasound techniques.

Cooperation: The studies were performed with international and Hungarian cooperation: (i) Department of Computer Science, Oxford, United Kingdom, (ii) Institute of Biomedical Engineering, Karlsruhe, Germany, (iii) Orion Pharma, Espoo, Finland, (iv) Department of Physiology, University of Debrecen, Hungary

Innovations: We developed and verified a novel NCX inhibitor compound, ORM-11372 cooperated with the Orion Pharma (Espoo, Finland).

Scientometrics :

Number of publications: 9 (7 original, 2 reviews; D1: 4, Q1: 4, Q3:1)

Cumulative impact factor: 56.17

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